[CANCER RESEARCH 48, 1361-1366, March 1, 1988]

Development of Highly Sensitive Immunoassays to Measure Human Chorionic Gonadotropin, Its β-Subunit, and β Core Fragment in the Urine: Application to Malignancies[1]

John F. O'Connor,[2] John P. Schlatterer, Steven Birken, Alexander Krichevsky, E. Glenn Armstrong,[3] Don McMahon, and Robert E. Canfield

Departments of Pathology [J. F. O., A. K., E. G. A.,] and Medicine [J. P. S., S. B., A. K., D. M., R. E. C.], College of Physicians and Surgeons of Columbia University, New York, New York 10032

ABSTRACT

A variety of malignancies have been associated with the presence of human chorionic gonadotropin, hCG, its subunits, and fragments of its β-subunit in blood and urine. The usefulness of these hCG-related tumor markers in nontrophoblastic malignancies has been inhibited by inadequate assay techniques. In order to achieve the required sensitivity and specificity, errors [illegible] cross-reacting human luteinizing hormone were necessary. In addition, the coexistence of a fragment of the hCG-β or β human luteinizing hormone subunit contributes to significant errors of measurement in urine. The importance of the hCG-β fragment as a potential tumor marker has been recognized previously but no method was available to measure this antigen readily. We report here the development of a series of radioimmunometric, two-site assays which will accurately measure hCG, hCG-β subunit, and the β-subunit fragment directly in small volumes of unprocessed urine. These assays are highly specific, extremely sensitive, and not labor intensive since they employ microtiter plate procedures. Application of these assays to urine samples from patients with gynecological malignancies indicated that over 50% of all patients tested excreted the hCG-β fragment in their urine. Also, this fragment comprised more than 50% of the moles of hCG immunoreactive components present in the specimens that were positive for hCG. This cancer marker is also demonstrable in trophoblastic malignant states such as choriocarcinoma in which the low molecular weight fragment can also be visualized directly by immunoblotting procedures. We conclude that a search for hCG immunoreactivity in the urine of patients with malignancies will be improved by the inclusion of accurate measurements of the prominent quantities of the β fragment excreted by these individuals.

INTRODUCTION

Sensitive serum measurements reflecting the production of hCG[4] by tumors became feasible with the report in 1972 by Vaitukaitis *et al.* (1) of an assay methodology for hCG based on an antiserum (SB-6) raised against the purified β-subunit of this hormone. This antiserum could detect intact hCG as well as its free β-subunit, present in body fluids or tissue extracts, without significant cross-reaction from hLH or other glycoprotein hormones with which hCG shares a common α-subunit. Shortly thereafter Braunstein *et al.* (2) employed this assay and showed that the hormone was secreted by a variety of nontrophoblastic, nontesticular neoplasms. Positive serum titers (≥1 ng hCG/ml serum) were found in 60 of 828 subjects tested (7.2%).

More sensitive measurements of hCG still required concentration and frequently chromatographic separations since, as the hLH components were concentrated, their cross-reactive contributions to total hCG immunoreactivity became unacceptably high (3-11). These tedious methods of analysis limited assays to a small number of specimens but did demonstrate that the excretion of small quantities of hCG was possible by normal, nonpregnant individuals, especially postmenopausal women (11, 12). The appearance of the hCG-free β-subunit or a fragment with β-subunit immunoreactivity in the urine has been reported by several investigators using concentration and separation techniques (5, 10, 13-15), and increased levels of circulating hCG and its subunits continued to be reported in a variety of malignancies (16, 17).

Measurement of hCG antigens in serum as compared to urine give disparate results. For example, a very recent report found little evidence for the widespread presence of intact hCG or free subunits in the serum of cancer patients (18). In contrast, the levels of hCG immunoreactive materials in the urine f patients with gynecological malignancies appear to be significantly higher than that found in their serum (5). Investigators have suggested that assay of urine for hCG-related tumor markers, especially the hCGβ fragment, may be more useful than serum assays (5, 10, 13). Therefore, we have undertaken to establish techniques to measure such antigens in the urine of cancer patients and the urine of pregnant women by simplified methods that retain a very high sensitivity and selectivity. Toward this goal we have developed a series of specific monoclonal (19, 20) and polyclonal (21) antibodies for two-site immunoassays which can specifically measure hCG, β-subunit, and a β-subunit fragment in unprocessed urine. In particular the contribution of this β fragment to total urinary hCG immunoreactivity has been shown to be very high by other investigators (4, 5, 10, 13). Therefore we have defined the structure of this fragment, prepared standard solutions, and developed antibodies to it (22, 23) to form the basis of one of the clinical assays described in this paper and a companion report by Cole *et al.* (24).

Some applications of these assays include the establishment of baseline measurement in normal, nonpregnant individuals (25), and the study of very early pregnancy (25, 26). Through the generosity of Dr. L. Cole of Yale University, we have also had the opportunity to evaluate the application of these urinary assays as markers of gynecological malignancy. The subject of this report is the description and validation of this methodology to measure these hCG antigens in the urine of cancer patients. The application of some f these meth ds to cancer specimens is described in a companion report (24).

MATERIALS AND METHODS

Reagents. The antibodies used in these studies are designated B108, B109, B201, B204, and R525. B108 is a monoclonal antibody which

Received 5/20/87; revised 11/19/87; accepted 12/2/87.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by NIH Grant P01HD-15454, NIEHS Contract NO-1-ES-4-5054, and NIH Grant RR00645.

[2] To whom requests for reprints should be addressed, at Columbia University College of Physicians and Surgeons, Clinical Research Center, PH 4-124, 630 West 168th Street, New York, NY 10032.

[3] Present address: Hybritech Incorporated, 11085 Torreyana Road, P. O. Box 269006(92126), San Diego, CA 92121.

[4] The abbreviations used are: hCG, human chorionic gonadotropin; hLH, human luteinizing hormone; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

binds to an epitope on the hCG-β-subunit located at or near the "SB-6" binding site (19, 20). B109 is a monoclonal antibody whose binding site requires that both the α- and β-subunits be together as the intact hCG molecule (23). B201 and 204 are monoclonal antibodies generated to the hCGβ fragment, and these bind to an epitope on the hCG-β-subunit which is masked in the intact hCG molecule (23). R525 is a polyclonal rabbit antiserum directed against the hCGβ CTP determinant (21). Detailed methodology regarding the preparation and characterization of each of these antibodies, as well as the basis on which binding sites have been assigned, is provided in the references cited.

Monoclonal antibodies were purified by affinity chromatography on protein A Sepharose (BioRad Maps II System) prior to use. The polyclonal antiserum, R525, was purified by affinity chromatography with an hCG-β Sepharose column (25). Iodination of the antibodies (20–50 μg) was accomplished with 1 mCi Na ^{125}I (Amersham, Arlington Heights, IL) using Iodogen (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions.

The standards employed in these studies were purified hCG (CR121 with a potency of 13,450 IU/mg *versus* Second International Standard), hCG-β-subunit (CR117) and hCG-α-subunit (CR119), which were described previously (27). hLH (hLH-I-1; AFP-4345-B, potency, [illegible] IU/mg) and hLH-β-subunit were gifts from the National Pituitary Agency, National Institute of Arthritis, Metabolism and Digestive Diseases, NIH. The purified hCG-β-subunit core fragment was prepared as noted by Birken *et al.* (22).

Construction and Performance of Immunoradiometric Assays. Competitive binding studies were performed with the antibodies noted earlier in order to determine which combinations could fulfill the essential requirement in the construction of an immunoradiometric assay, namely simultaneous binding of antibody pairs to hCG, free β-subunit, and hCG-β core fragment. A description of these methods appears elsewhere (19).

Simultaneous Assay for hCG, hCG-β, and β Fragment. Based on these data, antibodies B109 and B204 were chosen to be immobilized on a solid phase. They function to extract ("capture") intact hCG (B109) as well as both hCG-free β-subunit and β core fragment (B204). The radiolabeled detection antibody, B108, was chosen because it bound to an epitope common to all three of these molecular forms, and in each instance, could bind to them simultaneously with the two capture antibodies. Pretitered solutions of capture antibodies, B109 and B204, in 0.2 M sodium bicarbonate (pH 9.5) were mixed and frozen. Freezing of the antibody mixture was found to optimize the response. The antibody solution (200 μl in binding buffer, 0.2 M $NaHCO_3$, pH 9.5) was added to Immulon-2 microtiter strips (Fisher Scientific, Springfield, NJ) and incubated overnight at 4°C. The antibody solution was removed and any remaining binding sites blocked by the addition of 1% BSA containing 0.01% NaN_3. After the blocking solution had incubated for 3 h at room temperature (plates can be stored at 4°C for up to 4 weeks at this stage) the BSA was removed and the wells washed with deionized water. Eight standards, covering a range from 0.1 to 12.5 ng/ml were established in normal male urine. To the antibody-coated wells 200 μl of the standards or 200 μl of urine were added. The pH of both urine and standards was adjusted to 8.0 with 1 M Tris buffer, pH 9.0, prior to assay. Normal male urine was used to assess nonspecific binding. Standards and unknowns were run in duplicate.

The wells were incubated for 24 h at room temperature and washed with deionized water, and then 200 μl of iodinated antibody (40,000 cpm/tube) in assay buffer (phosphate buffered saline, 0.01 M EDTA, 0.01 M NaN_3, 0.1% bovine γ-globulin) were added. The wells were again incubated for 24 h at room temperature.

The unbound trace was aspirated and the wells washed five times with deionized water. Radioactivity of each was determined in a Packard gamma counter. A dose-response curve was generated by a spline function fit of the standards and the concentration of unknowns interpolated (28). The assay described quantifies intact hCG, free hCG-β-subunit, and β-subunit core fragment simultaneously. The results are expressed in moles of intact hCG.

Assays for Individual Measurement of hCG, hCG-β, and β Fragment. Assays for intact hCG, free hCG-β-subunit and β-subunit core fragment were constructed similarly, using the appropriate capture and detection antibody combinations, *i.e.*, B109-B108 for intact hCG, B201 for hCG-β-subunit and B108-B204 for the β fragment, respec Standard curves, covering the range of 0.097–12.5 ng/ml for hCG (B109-B108), 0.048–25 ng/ml for free hCG-β subunit (R525) and 0.002–0.54 pmol/ml for hCG-β-subunit core fragmen constructed using purified fractions of the appropriate antigen as ards. A standard curve was generated by a spline fit of the da unknowns values interpolated from this transform.

The urines analyzed in this study were provided by Dr. Lau Cole of Yale University (24) and were obtained from patients Hunter Gynecologic Oncology Clinic at Yale New Haven Ho The urine samples were collected in sterile containers and stored with the addition of proteolytic enzyme inhibitors. Urines to se control specimens were obtained from women with normal men function enrolled in studies designed to detect early fetal loss.

SDS-Gel Electrophoresis and Immunoblotting. SDS-gel electr resis was performed as a modification of the system observed by S in which hCG does not dissociate into its subunits (29). The sa were electroblotted and stained as described earlier (30).

RESULTS

Assay Methodologies and Statistical Evaluation

The combinations of monoclonal antibodies used in the struction of the immunoradiometric assays were chosen in instance on the basis of (*a*) their ability to bind simultane to the antigen being assayed; (*b*) their possessing the hig sensitivity for the detection of the antigen; and (*c*) lac significant cross-reaction with physiological levels of hom gous hormones likely to be present in the assay mixture.

The sensitivity (least detectable dose) and cross-react data for each of the immunoassays employed in this study presented in Table 1. All doses appear as pmol/ml of stan solutions. The assay for intact hCG uses B109 to capture complete hormone and B108 (anti-β) as the detection antib This assay reacts less than 1% with the other hCG r antigens. The free β-subunit assay uses B201 (anti-β, β f ment) to capture β components and R525 (anti-COOH term region) to detect only intact free β-subunit. Since the β CO terminal region is present in the β-subunit but absent in t fragment, R525 will recognize only the captured β-subunit not the β fragment, which has lost its COOH-terminal reg Two β fragment assays can be performed using B108 and B Assay I was used in the studies described in this paper bec of its lower cross-reactivity with hLH. Assay II would expected to yield similar results except for small discrepan resulting from different cross-reactivities with free β-sub and hLH. Lastly, the combination hCG component assay u B109 and B204 as capture antibodies and B108 detection simultaneously measure hCG, free β and β fragment to de a total hCG immunoreactivity measurement.

Typical standard curves obtained when the combination as for all components (B109/B204-B108) is run against in hCG, hCG-β, and hCG-β core fragment, are shown in Fig Each antigen generates a slightly different standard curve shown (Fig. 1). Measurements of an unknown sample mixt represent a value which is the sum of the contributi ns fr each of the curves.

Quality C ntrol Evaluati n of Immunoassays

Inter- and Intraassay Variati n. Intraassay validation determined by the method of Rodbard (31). Interassay variat was assessed by the cumulative coefficient of variation of n mal male urine pools to which known amounts of material b been added and by assay of serial dilutions of a pool of pr

Table 1 *Evaluation of cross-reactivities and sensitivities of immunoassays for hCG, its subunit, fragments and hLH*

	Intact hCG assay		β Fragment assay I		β Fragment assay II		β-Subunit assay		Combination hCG component assay	
	B109-B108[a]		B108-B204[a]		B204-B108[a]		B201-R525[a]		B109/B204-B108[a]	
	MDD[b]	CR[c] (%)	MDD	CR (%)	MDD	CR (%)	MDD	CR (%)	MDD	CR (%)
hCG	0.010	100	>2.7	<1	0.500	1	0.400	12	0.006	70
β frag	>2.0	<1	0.005	100	0.005	100	>2.00	<1	0.006	48
hCG-β	5.000	<1	0.030	15–25[d]	0.050	6	0.020	100	0.006	100
hLH	>3.3	<1	>3.3	<1	3.000	<1	>3.3	<1	>3.3	<1

[a] Radiolabeled immunoglobulins.
[b] MDD, minimum detectable dose in pmol/ml.
[c] CR, cross-reactivity; 100% denotes antigen detected with greatest sensitivity.
[d] At very high concentrations of free β-subunit, its cross-reactivity falls to lower levels.

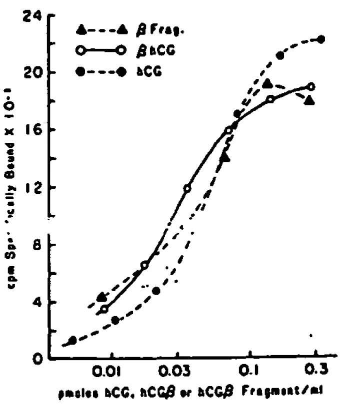

Fig. 1. Combination component assay for hCG, hCG-β, and hCG-β fragment. Standard curves obtained when intact hCG, hCG-β-subunit and hCG-β-subunit fragment are run in the combination hCG component assay which detects all hCG molecular species. *Abscissa*, pmol/ml of standard antigen solutions. The dose-response curves are similar but not identical and, thus, the quantitation of total hCG immunoreactive components in an unknown mixture is a close but not exact measurement.

Table 2 *Quality control data for immunoassays*

Assay	Interassay coefficient of variation (%)	Intraassay coefficient of variation (%)
Recoveries of antigenss added to male urine		
B109/B204-B108[a] total hCG components assay		
Intact hCG	8.2 (n = 44)	3.8
hCG free β-subunit	21 (n = 29)	
hCG β-subunit fragment	10 (n = 44)	
Serial dilution of pregnancy plasma		
C	16 (n = 44)	
C-1	18 (n = 40)	
C-2	14 (n = 33)	
C-3	14 (n = 34)	
hCG-β core fragment assay B108-B204	9.7 (n = 7)	6.7
hCG free β-subunit assay B201-R525[a]	14 (n = 3)	3.9
Intact hCG assay B109-B108[a]	7.4 (n = 5)	5.8

[a] Radiolabeled tracer antibodies.

nancy urine. The results of these determinations are presented in Table 2.

Recovery Studies. Recovery of hCG or subunits was determined by the addition of known quantities of material to normal male urine. In the (B109/B204-B108) combination assay, recovery of intact hCG averaged 98% (n = 11); of free hCG-β, 105% (n = 9); and of hCG-β core fragment 107% (n = 21).

In the B109-B108 assay for intact hCG recovery averaged 92% (n = 19); in the B108-B204 assay for hCG-β fragment recovery averaged 106% (n = 14); and in the B201-R525 assay for hCG-free β-subunit recovery averaged 79% (n = 9).

Assay Sensitivity

The minimum detectable dose, *i.e.*, the concentration of analyte resulting in an increase in cpm of bound tracer that was two standard deviations higher than the nonspecific binding (zero dose) binding, was 0.05 ng/ml (0.0014 pmol/ml) for the B109-B204-B108 combination assay (n = 8); 0.01 ng/ml (0.0014 pmol/ml) for the B108-B204 hCG-β core fragment assay (n = 12); 0.06 ng/ml (0.0016 pmol/ml) for the B109-B108 intact hCG assay (n = 8); and 0.05 ng/ml (0.0023 pmol/ml) for the B201-R525 assay for free hCG-β-subunit (n = 4). In actual practice, however, more conservative estimates of sensitivity (Table 1) were employed in assigning significance to patient samples.

Assay Specificity

Cross-reactivity studies are described in Table 1. The B109-B108 assay for intact hCG showed less than 1% cross-reaction with all the other antigens tested. The B201-R525 assay for the free hCG-β-subunit showed 12% cross-reactivity with intact hCG. The B108-B204 assay for hCG-β core fragment showed (15–25%) cross-reactivity with the free hCG-β subunit. Reversal of the capture and detection functions with these two antibodies resulted in a 6% cross-reactivity with free hCG-β-subunit. However, this system, while decreasing the contribution f hCG-β to measured immunoreactivity, showed increased cross-reaction with both intact hCG and hLH. Since the assay was riginally intended to be used in situations where appreciable levels of hCG and hLH might be present, the method as constructed for use by this laboratory is the B108-B204 version in order to limit this source of interference.

Clinical Application of Assay to Cancer Patients

Specimens were assayed by the several different immunoassays whose descriptions appear above. A total of 47 specimens were assayed by the combination hCG component assay designed to measure total immunoreactive hCG components (*i.e.*, intact hCG, hCG-free β-subunit, and hCG-β-subunit fragment) both collectively in a single assay and in separate assays, so that the contribution of each molecular species could be individually assessed.

Table 3 details the average molar contributions of intact

Table 3 *Average molar percentages of each hCG antigen in the urines of nontrophoblastic disease patients*

Antigen	Cervical	Endometrial	Ovarian	Other	Total
n	14	7	10	2	33
β Frag[a]	65	66	47	39	54
hCG-β[b]	11	1	1	6	5
Intact hCG[c]	24	33	53	55	41

[a] β Fragment assay I (B108-B204, radiolabeled immunoglobulin).
[b] β-Subunit assay (201-R525, radiolabeled immunoglobulin).
[c] Intact hCG assay (B109-B108, radiolabeled immunoglobulin).

Table 4 *Screening properties of immunoassays for hCG, its β-subunit, and β-subunit fragment when applied to normal women and women with gynecological cancers.*

	β Fragment	hCG-β	Intact hCG	Combination
Specificity of assays[a] (true negatives/all without disease, %)				
Normals[b]	68	78	21	21
Normals[c]	98	96	96	94
Sensitivity of assays[d] (true positives/all with disease, %)				
Cervical	55	20	40	70
Endometrial	86	29	86	100
Ovarian	40	7	47	47
Other	20	20	40	40
Total	51	17	49	64
Predictive value of a positive assay outcome (%)[e]				
Cervical	65	50	35	48
Endometrial	50	33	29	32
Ovarian	50	20	32	32
Other	14	20	12	12
Total	80	66	60	67
Predictive value of a negative assay outcome (%)[f]				
Cervical	59	47	25	40
Endometrial	93	73	80	100
Ovarian	59	50	33	33
Other	[illegible]	[illegible]	[illegible]	[illegible]
Total	36	26	14	19

[a] Epidemiological specificity is the property of a screening test to correctly identify true disease-free individuals. The higher the specificity the greater the proportion of true negatives to false positives.
[b] Postmenopausal women.
[c] Premenopausal women with multiple assays per individual, as follows: β fragment (13 women for 241 samples), hCG-β (18 women for 550 samples), intact hCG (18 women for 457 samples), and combination assay (61 women for 2191 samples).
[d] Epidemiological sensitivity is the property of a screening test to correctly identify true diseased individuals. The higher the sensitivity the greater the proportion of true positives to false negatives.
[e] Epidemiological positive predictive value is the proportion of positively screened individuals found to actually have the disease.
[f] Epidemiological negative predictive value is the proportion of negatively screened individuals found to actually be disease free.

hCG, hCG-free β-subunit, and hCG-β core fragment to the total molar mass of material present as a function of disease type. The hCG-β core fragment contributes more than 50% of the total mass overall. Specimens from 10 apparently healthy premenopausal women were run concurrently as controls. In no instance was a significant level of hormone or subunit measured in this population of normal individuals. A wider sampling of normal women is available from our studies of the incidence of early fetal loss in normal women attempting to conceive and in women undergoing artificial insemination.[5] The frequency of elevated levels obtained in a random set of nonconception cycles in the various assays is detailed in Table 4. It can be appreciated that the incidence of positive signal in any of the markers is on the order of 6% or less of the total samples assayed, indicating that the predictive value of a negative assay outcome is high and the test will therefore be of significant utility in this population group.

Although normal postmenopausal women, who are known to exhibit higher levels of hCG immunoreactivity (12, 25, 32, 33) in their urine, were not segregated from other subjects in the data analysis, a more profound problem with assay specificity might be anticipated in this group. An insight into the extent of this interference was gained by evaluating a group of 19 healthy postmen pausal w men with these assay systems. The presence of hCG immunoreactivity was confirmed in 15 f 19 subjects in both the c mbination assay and the intact hCG assay. Smaller numbers of positive results (f ur t six low level positives per assay) were btained in the subunit and fragment assays, suggesting that the intact hCG m lecule is the pred inant species in n rmal postmen pausal w men and there that assays which detect only this m lecule are f very lim utility in screening for malignant disorders. The impact f tl measurements on the efficiency of these assays as marker gynecological malignancy in postmenopausal women is sh in Table 4. These results have been calculated based upon values obtained from the postmenopausal w men only . therefore represent the worst-case scenario.

The combination assay proved to be the most sensitive (6 in the overall detection of malignant disease. However, hCG-β core fragment assay provided the highest verall posi predictive value, with 80% of positively tested individuals tually having some form of gynecological malignancy. T predictive value of a negative test value is much less usefu postmenopausal women than it would be expected t be premenopausal subjects, with the combination assay and core fragment assay accurately reflecting the absence of dise in only 19 and 36% of the subjects tested, respectively.

The specificity of monoclonal antibody B204 which is

[5] Performed in collaboration with Dr. Allen J. Wilcox and colleagues at NIEHS, Research Triangle Park, NC, and Dr. Wylie Hembree at Columbia University, New York, NY.

Fig. 2. Immunoblot analyses of hCG-β, β fragment, and urine samples fro choriocarcinoma patients. Samples were electrophoresed nonreduced in 18 SDS-polyacrylamide gels. Urine samples were desalted prior to application. Aft electrophoresis, gels were electrotransferred to nitrocellulose and stained wit monoclonal antibodies, B108 (*A*) and B204 (*B*). *Lanes 1* and *2*, β-subunit, 10, 2 pmol; *3* and *4*, β fragment, 10, 25 pmol; *5*, markers; *6*, *7*, and *8*, choriocarcinom urine samples. In *lanes 6* and *7*, the concentration of β fragment was very lo and difficult to visualize on the photograph. However, both *lanes 7* and *8* showe a band that corresponds in mobility with the standard β fragment in *lanes 3* an *4*. Note that B204 stained the hCG-β-subunit weakly while staining the β fragmen with high intensity. B108 stained the β fragment with equal intensity as the β subunit. This observation further supports the preference of B204 for recognitio of the β fragment over the intact β-subunit.

rected mostly t the β fragment is also demonstrated by the immunoblots in Fig. 2. In this experiment, the standard β fragment was electr phoresed (n nreduced gels in SDS) al ngside several urine samples from patients with choriocarcin ma (also supplied by Dr. Cole). The presence of high levels of β fragment can be visualized (by similar size to standard β fragment) while little or no free β-subunit (detected by B108) is apparent in these urine specimens.

DISCUSSION

Gynecological cancers have been estimated to comprise 28% of all cancers in women (34). The search for reliable tumor markers has included α-fetoprotein (16, 34, 35), carcinoembryonic antigen (36–38), and more recently, CA-125 for ovarian cancer (39, 40).

The primary utility of hCG as a tumor marker has been in the area of gestational trophoblastic disease, where it has been found useful not only as a disease marker but also as an indicator of therapeutic effectiveness (41). It is similarly useful in the diagnosis and treatment of testicular cancer (42, 43).

During the past two decades many improvements have been made in methods to measure hCG at low levels in serum. The principal problem of measurement of hCG by immunoassay is its homology to hLH, which may be present at high levels in normal individuals, especially close to the time of ovulation. Vaitukaitis *et al.* in 1972 (1) produced the first relatively specific immunoassay to distinguish hCG from hLH by using the hCG-β-subunit immunogen to generate the antiserum SB-6, but such highly discriminating antisera are an unusual occurrence. The β COOH-terminal portion of hCG has also been used as a hCG-specific antigen (it is absent in homologous hLH), but low affinity antibodies have usually been produced when synthetic peptides representing this region were employed as immunogens (44). Although the natural (glycosylated) peptide can produce higher affinity antibodies (21), it is costly to produce and must be combined with other antibodies for maximum sensitivity (23). Thus investigators have resorted to tedious concentration and separation procedures using serum or urine (3–15) to measure hCG at very low levels in these fluids. Such methods cannot be applied to large scale studies due to the labor and time involved. In addition, the presence of β fragments contribute to incorrect immunological estimates of hCG when hCG-β-directed antibodies are employed has also proven to be an important complication (10).

We report here the development of a series of immunoassays which overcome most of these problems and allow measurement of a variety of forms of hCG secreted in cancer patients' urine. The assays are not labor intensive and are extremely sensitive, specific, and not compromised by the presence of hLH or β fragment, which, in fact, can now be accurately measured. Since we have produced and characterized standard preparations of the β fragment (22) and have developed a series of monoclonal antibodies that can bind simultaneously to different sites on hCG or its β-subunit, it was possible to design the highly sensitive and specific assays described in this report. The assay designs all utilize the radioimmunometric or "sandwich" procedure (25) employing an immobilized capture antibody t extract the antigen from the biological fluid and a detection antibody with a radiolabel to measure the antigen that has become bound to the capture antibody.

Four specific immunoassays were designed to detect hCG r subunit components in unprocessed urine at sensitivities achieved earlier only by concentration and extraction procedures (3–15). These assays measure (*a*) intact hCG, (*b*) β fragment, (*c*) free β-subunit; and (*d*) all three components simultaneously, the latter being designated the combination hCG component assay (see Table 1 and Fig. 1). This combination assay which detects multiple species of hCG immunoreactivity offers a good appr ach for development of a single test for all the various types of hCG markers for cancer.

The β fragment assay system is unique since this is the first opportunity to use pure β fragment to construct standards that allow precise molar quantitation of the concentration of this important immunoreactive material in urine specimens fr m cancer patients. The assay can be conducted in two fashions by reversing the capture and detection antibodies. We have chosen the method which develops the lowest cross-reactivity with hLH.

Several investigations have documented the presence of circulating hCG in a variety of nontrophoblastic gynecological malignancies, with about 35% of patients with cervical and ovarian cancer estimated to have detectable serum levels (16, 45). A recent study by Papapetrou and Niconoulou (13) demonstrated the presence of urinary hCG-β core fragment in cancer patients (35–85% of total urinary immunoreactivity) but failed to find significant quantities of this molecule in extracts of tumors, where the predominant species was the free β-subunit. These investigators suggested assay of urine rather than the serum to assess these tumor markers (5, 13). These preliminary results obtained by the assay of the several urinary species of hCG reported here do indeed show pr mise of increasing the utility of hCG immunoreactive materials as markers in gynecological malignancy. In the companion report (1) which employs a different assay procedure, a finding of 74% positive results in patients with disease was obtained. Th se measurements detect both hCG-β core fragment and free hCG-β-subunit but do not distinguish between them. Our findings, using many of the same urines, indicate that the β fragment, rather than the complete hCG-β-subunit, is by far the predominant molecular species present in these specimens. Additi nal data must be accumulated in order to assign baseline values for screening of cancer patients. Nevertheless, it would appear that the presence of the hCG-β-subunit fragment as a significant urinary marker in gynecological malignancy has been established since 80% of positively tested individuals have some form of malignancy. The studies of others, especially the laboratories of Vaitukaitis, Schoeder, and Papapetrou (4, 5, 7, 10, 14, 15), support the presence and importance of the β fragment in a variety of nontrophoblastic malignancies. With the advent of this facile assay to measure large numbers of specimens, the patient population for study can readily be expanded to large sample numbers.

The occurrence of the hCG-β fragment is also supported by immunoblotting analyses of choriocarcinoma urines with the monoclonal antibodies B204 and B108. This study indicates that these urine samples contain high levels of β fragment (Fig. 2). The selectivity of antibody B204 for the β fragment as compared to its recognition of the free β-subunit is shown in this figure by the weak staining of free β in Fig. 2*B* (B204) when compared to the intense staining of β seen in Fig. 2*A* (B108).

In contrast to the results from cancer patients, ur urinary hCG determinations in early pregnancy, performed on samples obtained during the first 2 weeks post vulation (65 measurements in 14 pregnancies) sh w that the hCG-β-subunit fragment is present in am unts less than 15% of the t tal immun reactivity, with the remainder being pred minantly intact hCG. One rigin of the β fragment may be circulating free β-subunit as suggested by the infusion of Wehmann and Nisula

(33) which sh wed excreti n of β fragment in the urine f volunteers infused with β-subunit. Papapetr u *et al.* (13) showed that tum rs did n t contain the β fragment in patients with high levels f circulating β-subunit and β fragment in urine.

In summary, alth ugh the existence of the β core fragment and its applicability as a cancer cell marker in both nontrophoblastic as well as trophoblastic malignancies has been known for several years (4, 5, 6, 13–15), we now report a facile, rapid, and accurate method to measure this fragment in urine. This assay has the potential of immediate and important application in the diagnosis and management of certain types of cancers as reported here and in the accompanying manuscript by Cole *et al.* (24). It is important to use purified preparations of the hCG-β core fragment in the construction of these assays and monoclonal antibodies with specificities for its epitopes.

REFERENCES

1. Vaitukaitis, J. L., Braunstein, G. D., and Ross, G. T. A radioimmunoassay which specifically measures human chorionic gonadotropin in the presence of [illegible] Am. J. Obstet. Gynecol., *113:* 751–758, 1972.
2. Braunstein, G. D., Vaitukaitis, J. L., Carbone, P. O., and Ross, G. T. Ectopic production of human chorionic gonadotropin by neoplasms. Ann. Intern. Med., *78:* 39–45, 1973.
3. Schreiber, J. R., Rebor, R. W., Chen, H-C., Hodgen, G. D., and Ross, G. I. Limitation of the specific serum radioimmunoassay for human chorionic gonadotropin in the management of trophoblastic neoplasms. Am. J. Obstet. Gynecol., *125:* 705–707, 1976.
4. Hattori, M., Yoshimoto, Y., Matsukura, S., and Fujita, T. Quantitative and qualitative analyses of human chorionic gonadotropin and its subunits produced by malignant tumors. Cancer (Phila.), *45:* 355–361, 1980.
5. Papapetrou, P. D., Sakarelou, N. P., Braouzi, H., and Fessas, P. H. Ectopic production of human chorionic gonadotropin (hCG) by neoplasms: the value of measurement of immunoreactive hCG in the urine as a screening procedure. Cancer (Phila.), *45:* 2583–2592, 1980.
6. Wehmann, R. E., Ayala, A. R., Birken, S., Canfield, R. E., and Nisula, B. C. Improved monitoring of gestational trophoblastic neoplasia using a highly sensitive assay for urinary chorionic gonadotropin. Am. J. Obstet. Gynecol., *140:* 753–757, 1981.
7. Amr, S., Wehmann, R. E., Birken, S., Canfield, R. E., and Nisula, B. C. Characterization of a carboxyterminal peptide fragment of the human choriogonadotropin beta subunit excreted in the urine of a woman with choriocarcinoma. J. Clin. Invest., *71:* 329–339, 1983.
8. Borkowski, A., Puttaert, V., Gyling, M., Muquardt, C., and Body, J. J. Human chorionic gonadotropin-like substance in plasma of normal nonpregnant subject and women with breast cancer. J. Clin. Endocrinol. Metab., *58:* 1171–1178, 1984.
9. Javadpour, N., and Chen, H-C. Improved human chorionic gonadotropin detection with carboxyl-terminal radioimmunoassay of the beta subunit on concentrated 24-hour urine in patients with testicular cancer. J. Urol., *126:* 176–178, 1981.
10. Schroeder, H. R., and Halter, C. M. Specificity of human β-choriogonadotropin assays for the hormone and for an immunoreactive fragment present in urine during normal pregnancy. Clin. Chem., *29:* 667–671, 1983.
11. Robertson, D. M., Suginami, H., Hernandez Montes, H., Puri, C. P., Choi, S. K., and Dicafalusy, E. Studies on a human chorionic gonadotropin-like material present in non-pregnant subjects. Acta Endocrinol., *89:* 492–505, 1978.
12. Chen, H-C., Hodgen, G. D., Matsuura, S., Lin, L. J., Gross, E., Reichert, L. E., and Ross, G. T. Evidence for a gonadotropin from nonpregnant subjects that has physical immunological and biological similarities to human chorionic gonadotropin. Proc. Natl. Acad. Sci. USA, *73:* 2885–2889, 1976.
13. Papapetrou, P. D., and Nicopoulou, S. Ch. The origin of a human chorionic gonadotropin beta-subunit-core fragment excreted in the urine of patients with cancer. Acta Endocrinol. *112:* 415–422, 1986.
14. Masure, H. R., Jaffee, W. L., Sickel, M. A., Birken, S., Canfield, R. E., and Vaitukaitis, J. L. Characterization of a small molecular size urinary immunoreactive human chorionic gonadotropin (hCG)-like substance produced by normal placenta and by hCG-secreting neoplasms. J. Clin. Endocrinol. Metab., *53:* 1014–1020, 1981.
15. Vaitukaitis, J. L. Immunologic and physical characterization of human chorionic gonadotropin (hCG) secreted by tumors. J. Clin. Endocrinol. Metab., *37:* 505–514, 1973.
16. Donaldson, E. S., van Nagell, J. R., Pursell, S., Gay, E. C., Meeker, W. R., Kashmiri, R. and van de Voorder, J. Multiple biochemical markers in patients with gynecologic malignancies. Cancer (Phila.), *45:* 948–953, 1980.
17. Hussa, R. O. Human chorionic gonadotropin, a clinical marker: review of its biosynthesis. Ligand Rev., *3:* 1–43, 1981.
18. Ozturk, M., Bellet, D., Manil, L., Hennen, G., Frydman, R. and Wards, J. Physiological studies of human chorionic gonadotropin (hCG), α hCG and β hCG as measured by specific monoclonal immunoradiometric assays. Endocrinology, *120:* 549–558, 1987.
19. Ehrlich, P. H., Moustafa, Z. A., Krichevsky, A., Birken, S., Armstron[g,] G., and Canfield, R. E. Characterization and relative orientation of epi[topes] for monoclonal antibodies and antisera to human chorionic gonadotr[opin.] Am. J. Repro. Immunol. Microbiol., *8:* 48–54, 1985.
20. Ehrlich, P. H., Moyle, W. R., and Canfield, R. E. Monoclonal antibodi[es to] gonadotropin subunits. *In:* L. Birnbaumer and B. W. O'Malley (eds.), M[eth]ods in Enzymology, Vol. 109, pp. 638–655. New York: Academic P[ress,] 1985.
21. Birken, S., Canfield, R. E., Agosto, G., and Lewis, J. Preparation [and] characterization of an improved β-COOH-terminal immunogen for ge[nera]tion of specific and sensitive antisera to human chorionic gonadotr[opin.] Endocrinology, *110:* 1555–1563, 1982.
22. Birken, S., Kolks, M. A. G., Agosto, G. M., Armstrong, E. G., and Kric[hev]sky, A. The structure of the hCG β core fragment present in human pregn[ancy] urine. (Abstract 514). Endocrine Society Meeting, Anaheim, CA, 1986.
23. Armstrong, E. G., Krichevsky, A., Silverberg, S., Lustbader, J. W., Sch[lat]terer, J. P., Reich, L. L., and Canfield, R. E. Development of monocl[onal] antibodies to fragments of the β subunit of hCG for the detection of e[arly] pregnancy and the midcycle surge of hLH. (Abstract 199). Endocrine Soc[iety] Meeting, Anaheim, CA, 1986.
24. Cole, L. A., Wang, Y., Elliott, M., Latif, M., Chambers, J. T., Chambers, K., and Schwartz, P. E. Urinary human chorionic gonadotropin free subunit and β-core fragment: a new marker of gynecological cancers. Can[cer] Res., *48:* 1356–1360, 1988.
25. Armstrong, E. G., Ehrlich, P. H., Birken, S., Schlatterer, J. P., [illegible] Hembree, W. C., and Canfield, R. E. Use of a highly sensitive and spec[ific] immunoradiometric assay for detection of human chorionic gonadotropin [in] urine of normal, nonpregnant and pregnant individuals. J. Clin. Endocri[nol.] Metab., *59:* 867–873, 1984.
26. Wilcox, A. J., Weinberg, C. R., Wehmann, R. E., Armstrong, E. G., Canfi[eld,] R. E., and Nisula, B. C. Measuring early pregnancy loss, laboratory and fi[eld] methods. Fertil. Steril., *44:* 366–374, 1985.
27. Morgan, F. J., Canfield, R. E., Vaitukaitis, J. L., and Ross, G. T. Propert[ies] of the subunits of human chorionic gonadotropin. Endocrinology, *94:* 16[01–] 1606, 1974.
28. Wald, S. Spline functions in data analysis. Technometrics, *16:* 1, 1974.
29. Schlaff, S. An acrylamide gel recombination assay for glycoprotein horm[one] subunits. Endocrinology, *98:* 527–533, 1976.
30. Tobin, H. T., Staehlin, T., and Fordin, J. Electrophoretic transfer of prote[ins] from polyacrylamide gels to nitrocellulose sheets: procedure and some ap[pli]cations. Proc. Natl. Acad. Sci. USA, *76:* 4350–4354, 1979.
31. Rodbard, D. Statistical quality control and routine data processing [for] radioimmunoassays and immunoradiometric assays. Clin. Chem., *20:* 125[4–] 1270, 1974.
32. Stenman, U-H., Alfthan, H., Ranta, T., Vartiaiven, E., Jalkanen, J., a[nd] Seppala, M. Serum levels of human chorionic gonadotropin in nonpregn[ant] women and men are modulated by gonadotropin-releasing hormone a[nd] steroids. J. Clin. Endocrinol. Metab., *64:* 730–736, 1987.
33. Wehmann, R. E., and Nisula, B. C. Characterization of a discrete degrad[ation] product of the human chorionic gonadotropin beta subunit in human[s.] Clin. Endocrinol. Metab., *51:* 101–105, 1980.
34. Zornoza, J., Lewis, E., and Wallace, S. Radiologic techniques in the diagno[sis] and therapeutic management of gynecologic malignancies. *In:* A. A. For[tier] (ed.), Gynocologic Cancer, pp. 1–33. New York: Churchill Livingst[one,] 1984.
35. Seppala, M., and Ruoslahti, E. α-Fetoprotein: physiology and patholo[gy] during pregnancy and application to antenatal diagnosis. J. Perinat. Me[d.,] *1:* 104–113, 1973.
36. van Nagell, J. R. Gynecologic oncology. *In:* C. F. Griffith and A. F. Ful[ler] (eds.), Tumor Markers in Gynecologic Malignancies, pp. 63–79. Bosto[n:] Martinius Nijhoff, 1983.
37. Goldenberg, D. M., Sharkey, R. M., and Primus, F. J. Carcinoembryo[nic] antigen in histopathology immunooperoxidase staining of conventional t[u]mor section. J. Natl. Cancer Inst., *57:* 11–21, 1976.
38. van Nagell, J. R., Meeker, W., Parker, J. C., and Harralson, J. D. Car[ci]noembryonic antigen in patients with gynecologic malignancy. Canc[er] (Phila.), *35:* 1372–1376, 1975.
39. Bast, R. C., Feeney, M., Lazarus, H., Nadler, L. M., Colvin, R. B., a[nd] Knapp, R. C. Reactivity of a monoclonal antibody with human ovari[an] carcinoma. J. Clin. Invest., *68:* 1331–1337, 1981.
40. Niloff, J. M., Knapp, R. C., Schaetzl, E., Reynolds, C., and Bast, R. C. CA125 levels in obstetric and gynecologic patients. Obstet. Gynecol., *6[4:]* 703–707, 1984.
41. Cavanagh, D., Ruffolo, E. H., and Wardsen, D. E. Gestational Trophoblast[ic] Neoplasia A Clinicopathologic Approach, pp. 305–340. Norwalk, CT: Ap[]pleton-Century-Crofts, 1985.
42. Bosl, G. J., Geller, N. L., Cirrincione, C., Nisselbaum, J., Vogrin, D. Whitmore, W. F., and Golbey, R. B. Serum tumor markers in patients wi[th] metastatic germ cell tumors of the testis. Am. J. Med., *75:* 29–35, 1983.
43. Alm, P., Hultberg, B., and Olsson, A. M. Testicular tumor markers in tiss[ue] correlated to serum levels and clinical stage. Scand. J. Urol. Nephrol., *1[8:]* 283–288, 1984.
44. Chen, H-C., Matsurra, S., and Hrashi, M. Limitation and problems of HCG specific antisera. *In:* S. J. Segal (ed.), Chorionic Gonadotropin, pp. 231–[illegible] New York: Plenum Press, 1980.
45. Rutanen, E. M., and Seppala, M. The hCG-subunit radioimmunoassay [in] non-trophoblastic gynecologic tumors. Cancer (Phila.), *41:* 692–696, 19[78].